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<p>13. Abstract (Maximum 200 Words) <i>(abstract should contain no proprietary or confidential information)</i></p> <p>DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase involved in various DNA metabolism and damage signaling pathways. DNA damage activates DNA-PK, which in turn phosphorylates a number of key proteins involved in replication, repair, and transcription. Accordingly, DNA-PK has long been suspected as a factor involved in sensing and transmitting DNA damage signals to the downstream target, which eventually contributes to the genomic stability and prevention of cancer. The overall goal of this proposal is to explore the role of DNA-PK in the development and progression of breast cancer. Since DNA-PK is a DNA repair factor as well as involved in damage signaling pathway, levels of DNA-PK activity among breast cancer would contribute to their drug resistance and also provide the basis for selection of patients for treatment with chemotherapy drugs.</p> <p>From the first two years of our study, we concluded that a peptide-based inhibitor preventing DNA-PKcs from forming a complex with Ku70/Ku80 significantly lowered DNA-PK activity. Furthermore, treatment of these breast cancer cells with target peptide significantly lowered the cell growth only in the presence of ionizing radiation, indicating that the peptide-based inhibitor exhibited a positive effect of on lowering drug resistance by specifically targeting DNA-PK <i>in vivo</i>. This result also validates a physiologic role for DNA-PK in chemotherapy drug resistance of breast cancers.</p>				
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INTRODUCTION:

The overall goal of this proposal is to explore the role of DNA-dependent protein kinase (DNA-PK) in the development and progression of breast cancer. DNA-PK is a nuclear serine/threonine protein kinase involved in various DNA metabolism and damage signaling pathways. DNA damage activates DNA-PK, which in turn phosphorylates a number of key proteins involved in replication, repair, and transcription. Accordingly, DNA-PK has long been suspected as a factor involved in sensing and transmitting DNA damage signals to the downstream target, which eventually contributes to the genomic stability and prevention of cancer. To see a correlation between DNA-PK activity and different stages of cancer, DNA-PK activity levels of various stages of breast cancer will be analyzed. Outcome of this study will provide us the information as to whether DNA-PK can be used as a prognostic measure of breast cancer progression. We will also analyze breast cancer cells grown in culture and grown as tumor grafts in mice to see whether DNA-PK plays a role in chemotherapy resistance. A strong correlation between DNA-PK activity and drug resistance of breast cancers would provide the basis for selection of patients for treatment with chemotherapy drugs. Furthermore, the information regarding the role of DNA-PK in drug resistance would be very useful for drug discovery aimed at increasing the sensitivity of tumors to chemotherapy.

BODY:**Task 1. To characterize the relationship between DNA-PK and breast cancer development/progression (months 1-36)**

In order to assess DNA-PK activity and its expression in various stages of breast cancer cells, we obtained several tissue samples from the Indiana University Cancer Center (IUCC) Tumor Bank and carried out a preliminary study. The number of breast cancer tissues we tested, however, were only 5 samples that were not enough to make any conclusion on the relationship between DNA-PK and breast cancer development/progression. We still have some difficulty in obtaining enough number of specimens for different stages of breast cancer by the FIGO (Federation of International Gynecologic Organization) guideline, however the IU Cancer Center strongly supports our study and promised to provide enough number of specimens to carry out our proposed study. Once obtained, tissue samples will be grown in tissue culture dish (25 x 150 mm) and cell extracts will be prepared for measurement of DNA-PK activity. Also, molecular analysis of the alteration of DNA-PK activity in breast cancers will follow to evaluate the relationship between DNA-PK activity and the degree status of breast cancer.

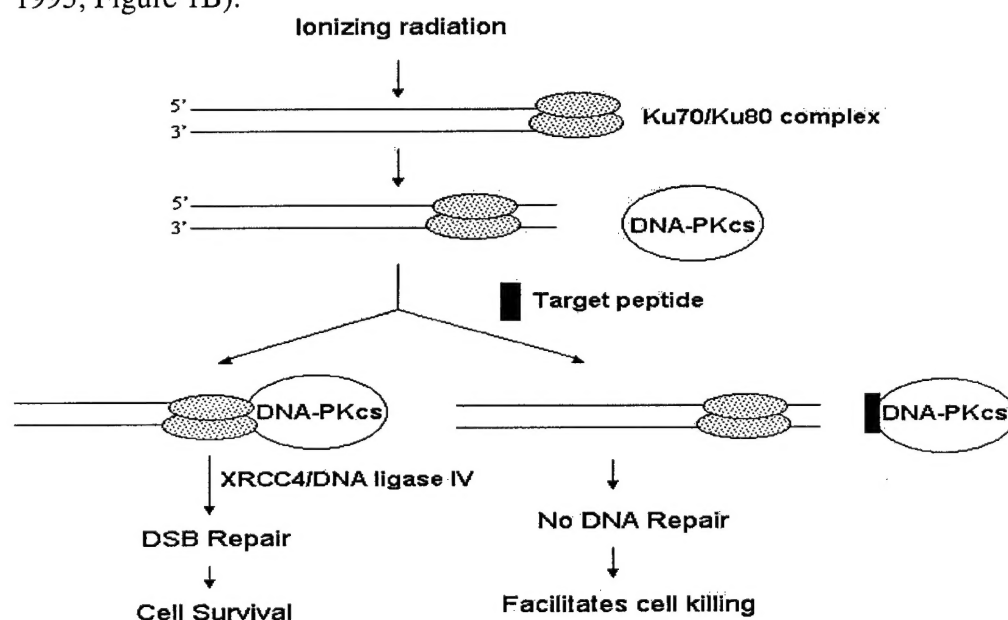
Task 2. To determine whether DNA-PK plays a role in DNA repair and/or the chemotherapy drug resistance among breast cancers (months 1-36)**DNA-PK activity and chemotherapy drug resistance of breast cancer cells (months 6-24):**

We initially proposed to determine whether altered DNA-PK activity in breast cancers is associated with chemotherapy drug. In order to see the relationship between DNA-PK and drug resistance of breast cancer cells, we prepared a peptide-based inhibitor that binds to the C-terminus of Ku80 (Gell & Jackson, 1999) and competitively inhibits DNA-PKs from binding to Ku70/Ku80. We tested these peptides for their effects on DNA-PK kinase activity *in vitro* using a specific substrate peptide (EPPLSQEAFADLWKK) (Lees-Miller *et al*, 1990). The target peptide (HNI-38) effectively inhibited DNA-PK activity under the conditions where the control peptide (HN-26) had no effect, indicating that HNI-38 prevented DNA-PKs from forming a complex with Ku70/Ku80 (Figure 2).

A previous study indicates that DNA-PK mutant cells exhibit sensitivity to irradiation and cisplatin treatment (Britten *et al*, 1999; Frit *et al*, 1999). Also, studies with drug-resistant and sensitive cells indicate that higher levels of DNA-PK expression lead to drug-resistant cells, whereas the low DNA-PK activity was associated with cells with drug-sensitive phenotype (Shen *et al*, 1997). Since the target peptide effectively interferes with DNA-PK activity, we attempted to analyze whether alteration of DNA-PK activity induced by treatment with peptide-based inhibitor affected drug resistance of breast cancer cells (Figs. 3-5).

Targeted Inhibition of DNA-PK

Ku70 and Ku80 form a heterodimeric complex that is important for DNA-termini binding; neither Ku70 nor Ku80 alone is active in DNA binding activity (Wu and Lieber, 1996; Gell and Jackson, 1999). The C-terminus of both Ku70 and Ku80 are necessary for heterodimer assembly as well as for DNA-termini binding (Wu and Lieber, 1996; Gell and Jackson, 1999). A recent protein interaction study indicated that DNA-PKcs interacting domain is localized at the extreme C-terminus of Ku80 (amino acids 720-732) (Gell and Jackson, 1999). Since the C-terminus of Ku80 is also likely involved in heterodimer assembly and DNA termini binding, this region (amino acids 720-732 of Ku80) was selected to synthesize a target peptide that would prevent DNA-PKcs from binding to Ku70/Ku80 regulatory subunits (see Figure 1A). To deliver a peptide to the cancer cells, a cell-permeable peptide import domain and the nuclear localization domain were added to the target peptide to obviate the need for permeabilization or microinjection of individual cells (Lin *et al*, 1995; Figure 1B).



HNI-38: AAVALLPAVLLALLAPVQRKRQKLMY-
 HN-26: AAVALLPAVLLALLAPVQRKRQKLMY
 HI-29: AAVALLPAVLLALLAPY-
 NI-22: VQRKRQKLMY-

Figure 1A (upper). A peptide co-therapy strategy for targeted inhibition of DNA-PK in cancer cell co-therapy. Treatment of cells with ionizing radiation (or chemotherapy drug) induces strand-break DNA

damage. To repair DNA damage, DNA-PK heterotrimeric complex (Ku70, Ku80, and DNA-PKcs) needs to be assembled at the ends of DNA. Target peptide representing amino acids 720-732 of Ku80 not only interacts with DNA-PKcs, but may also be involved in Ku heterodimer assembly and DNA termini binding. As a result, cells treated with target peptide will exhibit poor or no DNA repair and become highly sensitive to ionizing radiation or chemotherapy drug. **1B (lower)**. The Sequences of synthetic peptide-based inhibitor and control peptides (single-letter amino acid code). Membrane-translocating hydrophobic signal sequence is underlined and the nuclear localization sequence is shown in **bold face**. Twelve residue of peptide inhibitor region is indicated as bar (□) at the C-terminus. The tyrosine residue (Y) is included for 125 I-labeling to determine the import efficiency of the peptide into the cells. Peptide-based inhibitor contains the hydrophobic region localization sequence, so-called membrane-translocating carrier, which not only facilitates secretion of proteins, but also is important for importing synthetic peptides into the cell (Lin YZ *et al*, 1995). This localization peptide is capable of carrying a functional domain such as nuclear localization signal (NLS) (Boulikas T, 1994). We therefore synthesized a 38-residue peptide (HNI-38) comprising the signal peptide sequence (AAVALLPAVLLALLAP) and NLS (VQRKRQKLM) followed by a tyrosine (Y) residue and 12-residue of peptide inhibitor sequence (EGGDVDDLLDMI) representing the C-terminus of Ku80 (amino acids #721-732). The tyrosine residue was used for 125 I-labeling to determine the import efficiency of synthetic peptides into the cells (and nuclei) (Lin YZ *et al*, 1995).

A target peptide interrupts the interaction between DNA-PKcs and Ku70/Ku80 as well as the binding of Ku complex to DNA. DNA-PKcs and Ku70/Ku80 are abundant proteins approximately 5×10^5 molecules per human cells (Lee and Kim, 2002) and most of Ku70/Ku80 heterodimer exists in cell extracts without forming a complex with DNA-PKcs in the absence of DNA (Hammarsten and Chu, 1998). Therefore, target peptide (HNI-38) was analyzed for its effect on interaction between DNA-PKcs and Ku70/Ku80 in the presence of dsDNA. Varying concentrations of either control (HN-26) or target peptide (HNI-38) was incubated with cell extracts containing DNA-PKcs and Ku complex in the presence of dsDNA cellulose, and examined for its effect on binding of Ku complex and DNA-PKcs to DNA following the dsDNA cellulose pulldown assay (Figure 2A). Although it was marginal, the addition of increasing amount of target peptide (HNI-38) not control peptide (HN-26) led to a decrease in DNA-PKcs associated with dsDNA, suggesting that target peptide binds to DNA-PKcs and inhibits its binding to Ku70/Ku80. It is also noted that the addition of target peptide affected the binding of Ku70/Ku80 to the dsDNA cellulose (Figure 2A).

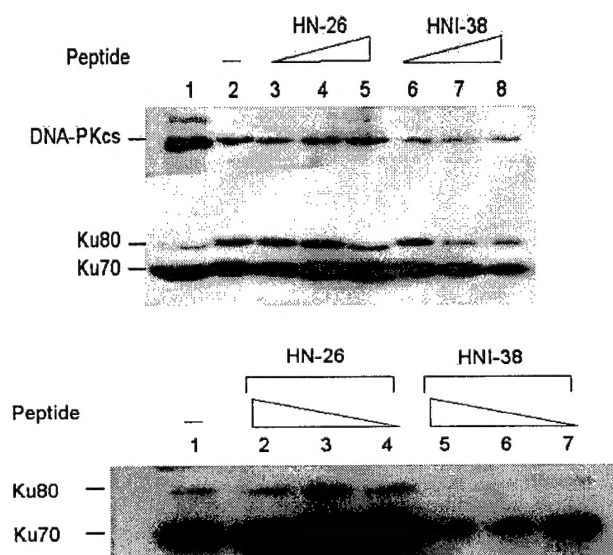


Figure 2. Effect of the target peptide on interaction of Ku70/Ku80 with DNA-PK or with dsDNA. **Panel A (upper).** The target peptide (HNI-38) interferes with association of DNA-PKcs with dsDNA. Partially purified DNA-PK fraction (100 ng) was incubated with 0 nM (lane 2), 10 nM (lanes 3 & 6), 50 nM (lanes 4 & 7), and 100 nM (lanes 5 & 8) of either control peptide or target peptide prior to dsDNA cellulose pull-down assay. Lane 1 contained partially purified DNA-PK without dsDNA pulldown assay. The protein-dsDNA cellulose complex was analyzed by the procedure described in Methods section. **Panel B (lower).** Effect of HNI-38 on DNA binding activity of Ku70/Ku80 complex. Purified Ku70/Ku80 complex (100 ng) was incubated with 10 nM (lanes 4 & 7), 50 nM (lanes 3 & 6), and 100 nM (lanes 2 & 5) of either control peptide or target peptide prior to dsDNA cellulose pull-down assay. Following the dsDNA cellulose pull-down, Ku70 and Ku80 were analyzed by 10% SDS-PAGE and Western blot.

To further examine the effect of HNI-38 on Ku's DNA binding activity, target peptide was incubated with purified Ku70/Ku80 complex in the presence of dsDNA cellulose, and the reaction mixtures were analyzed for the presence of Ku70 and Ku80 following the dsDNA pulldown assay (Figure 2B). In keeping with Figure 2A, target peptide (HNI-38) significantly interfered with binding of Ku complex to dsDNA under the conditions where control peptide (HN-26) showed virtually no effect (Figure 2B). This result suggests that target peptide not only affects the interaction between DNA-PKcs and Ku, but also interferes with Ku's DNA binding activity.

Effect of target peptide (HNI-38) on DNA-PK kinase activity

Interaction of DNA-PKcs with Ku complex are necessary for activation of its kinase activity (Gottlieb and Jackson, 1993; Hartley et al., 1995), therefore, the efficacy of target peptide was analyzed by measuring DNA-PK kinase activity *in vitro* in the presence of either HI-26 or HNI-38. DNA-PK kinase activity was inhibited up to 50% in the presence of HNI-38 under the conditions where a control peptide (HI-26) showed minimal effect (Figure 3), strongly supporting a notion that target peptide specifically binds to DNA-PKcs and interferes with interaction between DNA-PKcs and Ku complex. Inhibitory effect of target peptide on DNA-PK occurred at low peptide concentration ($>20 \mu\text{M}$) and, in the presence of $20 \mu\text{M}$ or higher, both target and control peptides inhibited DNA-PK activity (data not shown).

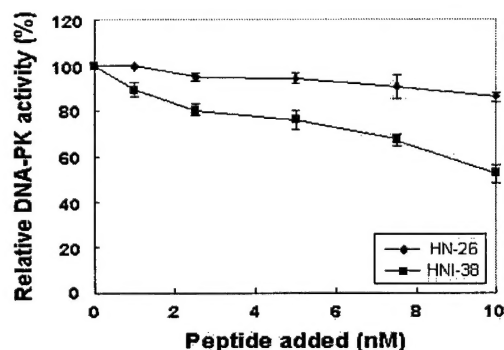


Figure 3. Effect of target peptide on DNA-PK kinase activity *in vitro*. Partially purified DNA-PK fraction was incubated with various concentrations of either control peptide or target peptide prior to the addition of substrate peptide and other components for DNA-PK kinase assay (see the Methods section for the details). DNA-PK activity was measured as the relative amounts of ^{32}P transferred to the substrate peptide.

Target peptide interferes with repair of double-stranded DNA breaks induced by IR

IR-induced double-stranded DNA breaks are efficiently repaired by non-homologous end-joining (NHEJ) process. Genetic and biochemical studies strongly indicated that DNA-PK plays an

essential role in NHEJ (Jeggo, 1998; Jin et al., 1997; Blunt et al., 1995). Hence, an alternative way to determine the efficacy of peptide inhibitor is to measure the repair of double-stranded DNA breaks following IR. Breast cancer cells (NCI) grown in the presence of [^{14}C]-thymidine (DiBiase et al, 2000) were treated with either a control or target peptide for 24 hrs. Following irradiation (40 Gy), cells were harvested at various time points and intact chromosomal DNA and DSBs were separated by pulsed field gel electrophoresis (0.5% agarose). Treatment of NCI cells with IR (40 Gy) induced substantial amounts of dsDNA breaks, most of which were repaired within 4 hrs. Cells treated with target or control peptide did not show any difference in generating DSBs following IR (Figure 4A; lane 2 vs. lanes 8 & 14). On the other hand, cells treated with target peptide (Figure 4A; lanes 14-18) compared with those treated with control peptide (Figure 4A; lanes 8-12) showed a noticeable decrease in DSB repair activity. This result suggests that target peptide interfered with dsb repair *in vivo* through the targeted inhibition of DNA-PK activity.

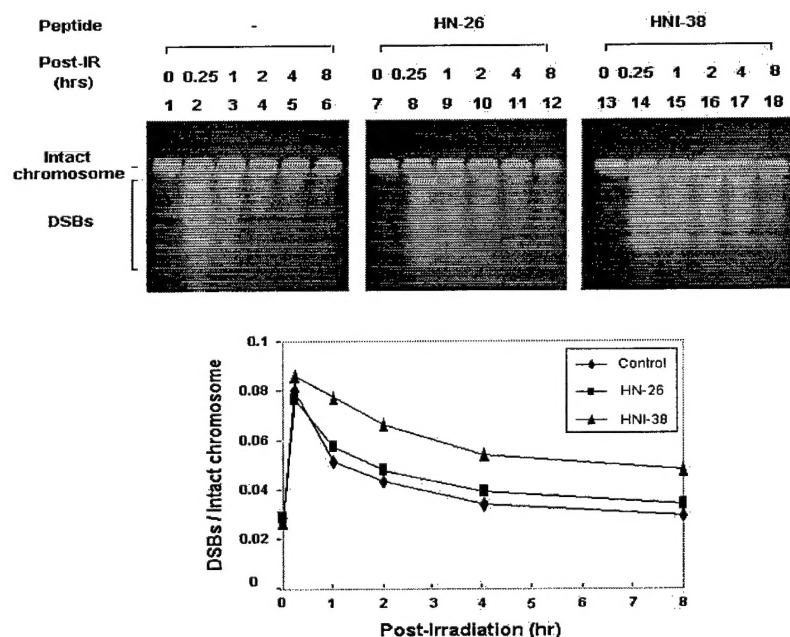


Figure 4. Effect of target peptide on double-stranded DNA breaks (dsb) repair. Breast cancer cells (NCI) grown in ^{14}C -containing media were treated with ionizing radiation (40 Gy) in the presence of no peptide (lanes 1-6), 50 nM of control peptide (lanes 7-12), or 50 nM of target peptide (lanes 13-18). After harvesting the cells at various time points, intact chromosomes and double-stranded DNA breaks (DSBs) were separated by gel electrophoresis (**upper panel**: fluorography) and were quantified by liquid scintillation counter (**lower panel**).

Target peptide inhibits breast cancer cell growth only in the presence of DNA damage

Cells lacking DNA-PK catalytic subunit showed increased sensitivity to DNA damaging drugs or ionizing radiation (IR) (Lees-Miller et al., 1995; Kirchgessner et al., 1995), suggesting that DNA-PK activity is essential for DNA repair and cell survival upon DNA damage. We therefore tested whether a targeted inhibition of DNA-PK by a peptide HNI-38 would sensitize breast cancer cells upon treatment of ionizing radiation or chemotherapeutic drug (cisplatin). Two breast cancer cells (NCI and MDA231) were treated with either control (HI-26) or target peptide (HNI-38) and tested for the efficacy of DNA-PK inhibitory peptide on lowering resistance of cells in response to ionizing

radiation using standard colony count cell survival assay. Both control and target peptides did not show any effect on cell growth in the absence of ionizing radiation. However, cells treated with IR showed significant cell growth inhibition in the presence of target peptide but not with control peptide (Figure 5A), suggesting that cell growth inhibition by target peptide occurs through targeting DNA-PK activity. Cells treated with cisplatin, although not as effective as those treated with IR, also showed inhibitory effect on cell growth in the presence of HNI-38 (Fig 5B).

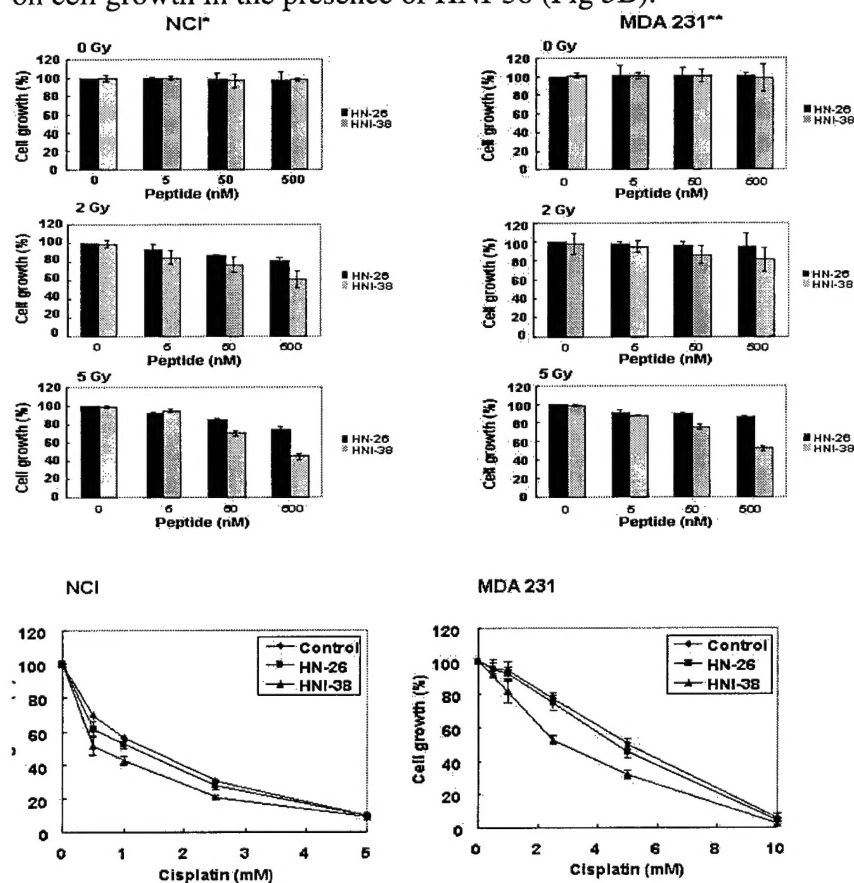


Figure 5. Effect of control (HN-26) and target (HNI-38) peptides on the growth of breast cancer cells treated with ionizing radiation (**upper panel**) or cisplatin (**lower panel**). Values expressed are means (+ S.E.) of the three replications (*, $p < 0.01$; **, $p < 0.01$). The clonogenic assay was used for the cells treated with ionizing radiation and the cell survival assay (MTT) was employed for those treated with cisplatin (see the Methods section for the detailed procedure).

In summation, DNA-PK activity is essential for DNA repair as well as cell cycle arrest in response to DNA damage, which contributes to cell survival by protecting cells from apoptosis. Cells treated with target peptide not control peptide showed a noticeable decrease in DSB repair following high dose (40 Gy) of IR, suggesting that HNI-38 specifically targets DNA-PK *in vivo* and interferes with dsb repair activity through inhibition of DNA-PK activity. Targeted inhibition of DNA-PK by HNI-38 also caused cell growth inhibition only when cells were treated with IR, suggesting that HNI-38 targeted DNA-PK and lowered resistance of cells in response to ionizing radiation, which eventually causes growth inhibition of both NCI and MDA231. Treatment of cells with HNI-38 also showed additive effect on cell growth inhibition in response to cisplatin treatment. This observation is in

keeping with previous findings that DNA-PK is directly involved in NER action in mammals (Muller et al., 1998). It also supports a notion that a targeted inhibition of DNA-PK would sensitize cancer cells upon treatment of chemotherapeutic drugs such as cisplatin. Together, our study described here not only validates DNA-PK as a useful molecular target for the treatment of drug-resistant cancer cells, but also supports physiologic role for DNA-PK in IR or chemotherapy drug resistance of cancer cells.

KEY RESEARCH ACCOMPLISHMENTS:

1. Demonstration of the relationship between DNA-PK activity and drug resistance of breast cancer cells
2. Validation of a peptide-based inhibitor of DNA-PK on lowering the growth of breast cancer cells following radiation treatment

REPORTABLE OUTCOMES:

1. Kim, C-H, Park, S-J, and Lee, S-H (2002) Sensitization of breast cancer cells by a targeted inhibition of DNA-dependent protein kinase. *J. Pharm. Exp. Ther.* (In press).
2. Invited presentation at the annual meeting for The Amelia Project (The Catherine Peachey Fund for Breast Cancer Research), Indianapolis, IN, February 2001.

CONCLUSIONS:

The overall goal of this proposal is to explore the role of DNA-PK in the development and progression of breast cancer. Since DNA-PK is a key DNA repair factor as well as involved in damage signaling pathway, levels of DNA-PK activity among breast cancer would contribute to their drug resistance and also provide the basis for selection of patients for treatment with chemotherapy drugs.

From the first two years of study, we concluded that a peptide-based inhibitor preventing DNA-PKs from forming a complex with Ku70/Ku80 significantly lowered DNA-PK activity. Furthermore, treatment of these breast cancer cells with target peptide significantly lowered the cell growth only in the presence of ionizing radiation, indicating that the peptide-based inhibitor exhibited a positive effect of on lowering drug resistance by specifically targeting DNA-PK *in vivo*. This result also validates a physiologic role for DNA-PK in chemotherapy drug resistance of breast cancers.

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APPENDICES:

Manuscript in press (Kim *et al*)

A targeted inhibition of DNA-dependent protein kinase sensitizes breast cancer cells following ionizing radiation†

Chung-Hui Kim, Su-Jung Park, and Suk-Hee Lee*

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Running title: A novel cancer co-therapy targeting a key DNA repair enzyme, DNA-PK

(Key words: DNA-dependent protein kinase (DNA-PK), Ku70/Ku80 complex, ionizing radiation, breast cancer cells, DNA repair, peptide co-therapy)

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ABBREVIATIONS

AFIGE, asymmetric field inversion gel electrophoresis; BER, base excision repair; DMEM, Dulbecco's minimal essential medium; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, catalytic subunit of DNA-dependent protein kinase; Dsbs, double-stranded DNA breaks; DTT, dithiothreitol; IR, ionizing radiation; MDA231, a human breast cancer cell lines; MTT, 3-(4, 5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide; NCI, a human breast cancer cell lines; NER, nucleotide excision repair; NHEJ, non-homologous end-joining; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; SCID, severe combined immune deficiency mouse cells; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

ABSTRACT

A major mechanism by which cancer cells become resistant to ionizing radiation (IR) and chemotherapy drugs is by enhanced DNA repair of the lesions, therefore, through inhibition of DNA repair pathways that tumor cells rely on to escape chemotherapy, we expect to increase the killing of cancer cells and reduce drug resistance. DNA dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase essential for DNA repair as well as sensing and transmitting damage signal to downstream targets leading to the cell cycles arrest. We used a peptide co-therapy strategy to see whether a targeted inhibition of DNA-PK activity sensitizes breast cancer cells in response to IR or chemotherapy drug. A synthesized peptide representing the C-terminus of Ku80 (HNI-38) selectively targeted and disrupted interaction between Ku complex and DNA-PKcs as well as Ku's DNA binding activity that led to the inhibition of DNA-PK activity and reduction in double-stranded DNA break (dsb) repair activity. Furthermore, a peptide-based inhibitor with target sequence effectively inhibited the growth of breast cancer cells only in the presence of DNA damage, suggesting that the target peptide sensitizes cancer cells through blocking dsb DNA repair activity. Together, this study not only validates the involvement of C-terminus of Ku80 in Ku's DNA termini binding and interaction with DNA-PKcs, but also supports physiologic role for DNA-PK in IR or chemotherapy drug resistance of cancer cells.

INTRODUCTION

DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase composed of 460-kDa catalytic subunit (DNA-PKcs) and a heterodimer of Ku70 and Ku80, which act as a DNA binding and regulatory component for the complex (Gottlieb and Jackson, 1993; Jin et al., 1997; Lieber et al., 1997). DNA-PK is a key component of the non-homologous end joining (NHEJ) pathway and V(D)J recombination (Blunt et al., 1995) with the unique property of being activated by DNA ends (Jeggo, 1998; Featherstone and Jackson, 1999; Critchlow and Jackson, 1998). It has long been suspected as a factor involved in sensing and transmitting DNA damage signals to the downstream targets (Weaver and Alt, 1997; Jackson, 1997; Lee and Kim, 2002). Previous studies demonstrated that DNA-PK is necessary for activation of p53 (Woo et al., 1998), nucleotide excision repair (Muller et al., 1998), and damage-induced S-phase arrest (Park et al., 1999) in response to DNA damage, all of which contribute to cell protection from genetic alterations as well as chemotherapy drug resistance. *In vivo* observations indicated that DNA-PK mutant cells exhibited sensitivity to ionizing irradiation and chemotherapy drugs and were associated with lower DNA repair activity following DNA damage, suggesting a positive role for DNA-PKcs in DNA repair (Britten et al., 1999; Frit et al., 1999). Also, studies with drug-resistant or drug-sensitive cancer cells suggested that higher levels of DNA-PK expression lead to a drug-resistant cells, whereas the low DNA-PK activity was associated with cells with drug-sensitive phenotype (Shen et al., 1997) and was linked to cell death via the accumulation of damaged DNA.

The current model of DNA-PK complex activation by DNA is based on the tenet that without DNA, DNA-PKcs is inactive and incapable of binding Ku (Hanawalt, 1994; Suwa et al., 1994; Hartley et al., 1995). When a double-strand break is introduced, Ku complex

binds to the DNA because of its high affinity for DNA ends. The binding of Ku induces conformational change that allows it to interact with DNA-PKcs. It is unclear how the Ku/DNA complex activates the kinase activity of DNA-PKcs. One hypothesis is that DNA-PKcs undergoes a conformational change upon association with the Ku/DNA complex and this conformational change accounts for the activation of kinase activity. The kinase activity associated with DNA-PK is needed for DNA repair *in vivo*, since expression of a kinase-inactive form of DNA-PKcs failed to complement the radiosensitive phenotype of a mammalian cell line lacking the DNA-PKcs protein (Kurimasa et al., 1999). However, the physiological targets of DNA-PK *in vivo* are still not clear. The DNA-PK complex can physically tether two ends of a DSB in close proximity *in vitro*, suggesting the hypothesis that the DNA-PK complex acts as a scaffold to assemble the NHEJ pathway proteins at a DSB (Cary et al., 1997).

We hypothesize that (i) DNA-PK plays an important role in conferring cells becoming resistance to ionizing radiation or anticancer DNA damaging drugs, and (ii) targeted inhibition of DNA-PK sensitizes drug-resistance of cancer cells and facilitates cell killing. By developing peptides that can directly interfere with DNA-PK activity, one can develop a novel co-therapy that can selectively target and disrupt IR-induced dsb repair pathway, which will enhance the efficacy of currently available treatments and also broaden the usefulness of chemotherapeutic agents in cancer treatment. We have therefore synthesized a peptide (HNI-38) mimicing the domain of Ku80 essential for interaction with its catalytic subunit (DNA-PKcs) and tested whether it can selectively target and disrupt DNA-PK activity required for dsb repair, which potentiates the effect of chemotherapy drug in cancer treatment. This strategy can be applied to cancer co-therapy, which will broaden the usefulness of chemotherapeutic agents in cancer treatment.

METHODS

Cell lines, antibodies, and chemicals. Two human breast cancer cells, MDA231 and NCI, were obtained from Dr. George Sledge (Indiana University Cancer Center, Indianapolis, IN), and maintained in MEM supplemented with 10 % fetal bovine serum at 37°C in a CO₂ incubator. Antibodies to Ku70/80 and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) were obtained from either Upstate Biotechnology (Lake Placid, NY) or Pharmingen (San Diego, CA). [γ -³²P]ATP (4500 Ci/mmol) was from ICN, and dsDNA cellulose and cisplatin were obtained from Sigma Chemical Co. (St. Louis, MO).

dsDNA cellulose pull-down assay. The dsDNA cellulose fraction (100 μ g) containing DNA-PKcs and Ku70/Ku80 heterodimer were prepared from HeLa cells (Lees-Miller et al., 1990) and incubated with indicated amount of either control (HN-26) or a target (HNI-38) peptide in the presence of 4 mM ATP and 50 μ l of dsDNA cellulose (3mg of dsDNA/mg cellulose, Sigma Chemicals, St. Louis, Mo) for 3 hr at 4°C with rocking for the interaction of DNA-PKcs and Ku70/Ku80 heterodimer. Where indicated, purified Ku70/Ku80 complex (100 ng) was used instead of the dsDNA cellulose fraction. After centrifugation at 4000 rpm, the precipitates were collected and washed three times with a buffer (50 mM Tris-HCl, pH8.0, 200 mM NaCl, 0.5% Nonidet p-40, 1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.1 mM PMSF) for protein analysis. For Western blot, the precipitates were separated by 8 or 10% SDS-PAGE, transferred to nitrocellulose (Millipore), blotted with primary antibody to Ku70/80 and/or DNA-PKcs followed by a peroxidase-coupled secondary antibody (Amersham) and an enhanced chemiluminescence (ECL kit, Amersham) reaction prior to visualization on Kodak-o-mat film.

DNA-PK kinase assay. Reaction mixtures (20 μ l) contained 20 mM HEPES-KOH (pH7.5), 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 7 mM $MnCl_2$, 5 mM NaF, 1 mM Na_3VO_4 , 50 μ l of ^{32}P -ATP, 150 μ l of substrate peptide, 0.4 μ g of DNase I-activated calf-thymus DNA (Sigma Chemical Co., St. Louis, MO), and 100 ng of partially purified DNA-PK complex. DNA-PK complex was partially purified from HeLa cells according to the procedure described previously (Lees-Miller et al., 1990). Substrate peptide (EPPL**SQ**EAFADLWKK) representing amino acids #11-24 of p53 was used as a substrate for DNA-PK assay (Lees-Miller et al., 1990). To find out whether the peptide interferes with DNA-PK kinase activity, various amounts of peptide inhibitor was added to the reaction. After incubation at 30°C for 30 min, the reaction mixtures were stopped with 30% acetic acid and a portion of the reaction mixtures (5 μ l) will be spotted onto a P81-strip and after extensive washing radioactivity was measured. DNA-PK activity was measured as pmol of ^{32}P transferred to the substrate peptide.

Cell survival assay. Cells (1.0×10^4 cells/ well) were seeded in a 96 well plate in the presence of control or target peptide and incubated for 24 hrs prior to the treatment of cells with either ionizing radiation or cisplatin. After further incubation at 37°C 5% CO_2 for 72 hrs, cell survival was measured using a colorimetric cell survival assay from Boehringer Mannheim (MTT Cell Proliferation Kit). Alternatively, clonogenic assay was used to measure the ability of cells to form colonies on 100 mm^2 tissue culture dishes following treatment with ionizing radiation or cisplatin. Controls consisted of cells untreated with peptides, DNA damaging agent, or with neither. Cells were continuously exposed for 5 days to the indicated concentrations of the peptide, and colonies stained with crystal violet, then colonies greater than 50 cells were counted. Each point represents mean values \pm SE, each conducted with triplicate plates. The *p*-values in Figure 5A were obtained from two

separate experiments using one way ANOVA method (SigmaStat for Windows, version 2.03).

Double-stranded DNA break (dsb) repair assay. Kinetics of rejoining of radiation-induced damaged DNA in breast cancer cells following exposure of cells to 40 Gy gamma irradiation (^{137}Cs) were measured by pulsed field gel electrophoresis. Breast cancer cells (NCI) were grown in the presence of $2.5\ \mu\text{M}$ [^{14}C]-thymidine ($0.1\ \mu\text{Ci/ml}$) (DiBiase et al., 2000) and treated with either a control or target peptide. Following irradiation (40 Gy), cells were further incubated at 37°C with prewarmed (42°C) fresh medium to allow DSB repair and then harvested at various times and resuspended in serum-free medium at a concentration of 2.5×10^6 cells/ml. Cells were mixed with an equal volume of 1% agarose and the solidified cell-agarose suspension were lysed with buffer containing 10 mM Tris (pH 8.0), 50 mM NaCl, 0.5 M EDTA, 2% N-lauryl sarcosyl, and proteinase E & O ($0.1\ \text{mg/ml}$) for 16-18 hr at 50°C (DiBiase et al., 2000). DNA double-strand breaks were analyzed by asymmetric field inversion gel electrophoresis (AFIGE) using 0.5% agarose gel in 0.5X TBE at 10°C for 40 hr. After electrophoresis, gels were analyzed by fluorography. For quantification of damaged DNA repair, intact chromosome and damaged DNA were separately removed from gel and measured for [^{14}C] using liquid scintillation counter.

RESULTS

Targeted Inhibition of DNA-PK

Ku70 and Ku80 form a heterodimeric complex that is important for DNA-termini binding; neither Ku70 nor Ku80 alone is active in DNA binding activity (Wu and Lieber, 1996; Gell and Jackson, 1999). The C-terminus of both Ku70 and Ku80 are necessary for heterodimer assembly as well as for DNA-termini binding (Wu and Lieber, 1996; Gell and Jackson, 1999). A recent protein interaction study indicated that DNA-PKcs interacting domain is localized at the extreme C-terminus of Ku80 (amino acids 720-732) (Gell and Jackson, 1999). Since the C-terminus of Ku80 is also likely involved in heterodimer assembly and DNA termini binding, this region (amino acids 720-732 of Ku80) was selected to synthesize a target peptide that would prevent DNA-PKcs from binding to Ku70/Ku80 regulatory subunits (see Figure 1A). To deliver a peptide to the cancer cells, a cell-permeable peptide import domain and the nuclear localization domain were added to the target peptide to obviate the need for permeabilization or microinjection of individual cells (Lin et al., 1995; Figure 1B).

A target peptide interrupts the interaction between DNA-PKcs and Ku70/Ku80 as well as the binding of Ku complex to DNA

DNA-PKcs and Ku70/Ku80 are abundant proteins approximately 5×10^5 molecules per human cells (Lee and Kim, 2002 and the ref. therein) and most of Ku70/Ku80 heterodimer exists in cell extracts without forming a complex with DNA-PKcs in the absence of DNA (Hammarsten and Chu, 1998; Figure 2A). Therefore, target peptide (HNI-38) was analyzed for its effect on interaction between DNA-PKcs and Ku70/Ku80 in the presence of dsDNA. Varying concentrations of either control (HN-26) or target peptide (HNI-38) was incubated

with cell extracts containing DNA-PKcs and Ku complex in the presence of dsDNA cellulose, and examined for its effect on binding of Ku complex and DNA-PKcs to DNA following the dsDNA cellulose pulldown assay (Figure 2B). Although it was marginal, the addition of increasing amount of target peptide (HNI-38) not control peptide (HN-26) led to a decrease in DNA-PKcs associated with dsDNA, suggesting that target peptide binds to DNA-PKcs and inhibits its binding to Ku70/Ku80. It is also noted that the addition of target peptide affected the binding of Ku70/Ku80 to the dsDNA cellulose (Figure 2B). To further examine the effect of HNI-38 on Ku's DNA binding activity, target peptide was incubated with purified Ku70/Ku80 complex in the presence of dsDNA cellulose, and the reaction mixtures were analyzed for the presence of Ku70 and Ku80 following the dsDNA pulldown assay (Figure 2C). In keeping with Figure 2B, target peptide (HNI-38) significantly interfered with binding of Ku complex to dsDNA under the conditions where control peptide (HN-26) showed virtually no effect (Figure 2C). This result suggests that target peptide not only affects the interaction between DNA-PKcs and Ku complex, but also interferes with Ku's DNA binding activity.

Effect of target peptide (HNI-38) on DNA-PK kinase activity

Interaction of DNA-PKcs with Ku complex are necessary for activation of its kinase activity (Gottlieb and Jackson, 1993; Hartley et al., 1995), therefore, the efficacy of target peptide was analyzed by measuring DNA-PK kinase activity *in vitro* in the presence of either HI-26 or HNI-38. DNA-PK kinase activity was inhibited up to 50% in the presence of HNI-38 under the conditions where a control peptide (HI-26) showed minimal effect (Figure 3), strongly supporting a notion that target peptide specifically binds to DNA-PKcs and interferes with interaction between DNA-PKcs and Ku complex. Inhibitory effect of target peptide on DNA-PK occurred at low peptide concentration ($>20 \mu\text{M}$) and, in the presence of $20 \mu\text{M}$ or higher, both target and control peptides inhibited DNA-PK activity (data not shown).

Target peptide interferes with repair of double-stranded DNA breaks induced by IR

IR-induced double-stranded DNA breaks are efficiently repaired by non-homologous end-joining (NHEJ) process. Genetic and biochemical studies strongly indicated that DNA-PK plays an essential role in NHEJ (Jeggo, 1998; Jin et al., 1997; Blunt et al., 1995). Hence, an alternative way to determine the efficacy of peptide inhibitor is to measure the repair of double-stranded DNA breaks following IR. Breast cancer cells (NCI) grown in the presence of [¹⁴C]-thymidine (DiBiase et al, 2000) were treated with either a control or target peptide for 24 hrs. Following irradiation (40 Gy), cells were harvested at various time points and intact chromosomal DNA and DSBs were separated by pulsed field gel electrophoresis (0.5% agarose). Treatment of NCI cells with IR (40 Gy) induced substantial amounts of dsDNA breaks, most of which were repaired within 4 hrs. Cells treated with target or control peptide did not show any difference in generating DSBs following IR (Figure 4A; lane 2 vs. lanes 8 & 14). On the other hand, cells treated with target peptide (Figure 4A; lanes 14-18) compared with those treated with control peptide (Figure 4A; lanes 8-12) showed a noticeable decrease in DSB repair activity. This result suggests that target peptide interfered with dsb repair *in vivo* through the targeted inhibition of DNA-PK activity.

Target peptide inhibits breast cancer cell growth only in the presence of DNA damage

Cells lacking DNA-PK catalytic subunit showed increased sensitivity to DNA damaging drugs or ionizing radiation (IR) (Lees-Miller et al., 1995; Kirchgessner et al., 1995), suggesting that DNA-PK activity is essential for DNA repair and cell survival upon DNA damage. We therefore tested whether a targeted inhibition of DNA-PK by a peptide HNI-38 would sensitize breast cancer cells upon treatment of ionizing radiation or chemotherapeutic drug (cisplatin). Two breast cancer cells (NCI and MDA231) were treated with either control (HI-26) or target peptide (HNI-38) and tested for the efficacy of DNA-PK inhibitory peptide on

lowering resistance of cells in response to ionizing radiation using standard colony count cell survival assay. Both control and target peptides did not show any effect on cell growth in the absence of ionizing radiation. However, cells treated with IR showed significant cell growth inhibition in the presence of target peptide but not with control peptide (Figure 5A), suggesting that cell growth inhibition by target peptide occurs through targeting DNA-PK activity. Cells treated with cisplatin, although not as effective as those treated with ionizing radiation, also showed inhibitory effect on cell growth in the presence of HNI-38 (Figure 5B).

DISCUSSIONS

Many key human DNA repair pathways, such as double-strand break repair or nucleotide excision repair pathway, rely on multimeric polypeptide activities (Wood, 1996; Sancar, 1996; Friedberg, 1996; Lee, 2001). Interactions between damage recognition proteins and those proteins that report the damage to downstream repair activities are crucial for DNA repair. DNA-PK is a key component of the non-homologous end joining (NHEJ) pathway with the unique property of being activated by double-stranded DNA breaks (Blunt et al., 1995). Earlier studies with drug-resistant and -sensitive cancer cells suggested that high level expression of DNA-PK leads to drug-resistant cells, whereas low DNA-PK activity was associated with drug-sensitive phenotype (Muller and Salle, 1997; Shen et al., 1997; Muller et al., 1998; Tew et al., 1998; Shen et al., 1998; Frit et al., 1999; Kim et al., 1999; Kim et al., 2000), implicating a role for DNA-PK in conferring cells becoming drug resistance in response to anticancer DNA damaging drug. Since the interaction of DNA-PKcs to its regulatory subunits, Ku70/Ku80, is crucial for its function in DNA repair, a targeted inhibition of DNA-PK would sensitize drug-resistance of cancer cells and facilitates cell killing. Therefore, we attempted to develop a peptide co-therapy strategy that a low molecular weight peptide-based inhibitor specifically interferes with interaction between DNA-PK catalytic subunit (DNA-PKcs) and Ku complex.

A target peptide (HNI-38) containing the C-terminus of Ku80 interfered with the interaction between DNA-PKcs and Ku complex. This was much an anticipated result since the C-terminus of Ku80 was previously identified as DNA-PKcs interacting domain (Gell and Jackson, 1999). Inhibitory effect of HNI-38 on the interaction between DNA-PKcs and Ku70/Ku80 directly affected its kinase activity, showing inhibition of DNA-PK activity up to 50% under the conditions where a control peptide (HI-26) showed very little effect (Figure

3). Addition of excess amount of target peptide however did not show any further inhibition of DNA-PK kinase activity (data not shown). This is likely due to the fact that DNA-PKcs without Ku complex can still function as a kinase although its activity is low. A target peptide (HNI-38) not only inhibited the interaction of DNA-PKcs with Ku complex on dsDNA, but also affected the Ku's dsDNA binding activity (Figure 2C). It is not clear how HNI-38 interferes with DNA binding activity of the Ku complex, however, the C-terminus of both Ku70 and Ku80 has been shown to be important for heterodimer assembly as well as for DNA-termini binding (Wu and Lieber, 1996; Gell and Jackson, 1999). It is possible that HNI-38 may interfere with the Ku70-Ku80 interaction through its binding to Ku70, which would negatively influence Ku's DNA-termini binding activity.

DNA-PK activity is essential for DNA repair as well as cell cycle arrest in response to DNA damage, which contributes to cell survival by protecting cells from apoptosis. Cells treated with target peptide not control peptide showed a noticeable decrease in DSB repair following high dose (40 Gy) of IR, suggesting that HNI-38 specifically targets DNA-PK *in vivo* and interferes with dsb repair activity through inhibition of DNA-PK activity. Targeted inhibition of DNA-PK by HNI-38 also caused cell growth inhibition only when cells were treated with IR, suggesting that HNI-38 targeted DNA-PK and lowered resistance of cells in response to ionizing radiation, which eventually causes growth inhibition of both NCI and MDA231. Treatment of cells with HNI-38 also showed additive effect on cell growth inhibition in response to cisplatin treatment. This observation is in keeping with previous findings that DNA-PK is directly involved in NER action in mammals (Muller et al., 1998). It also supports a notion that a targeted inhibition of DNA-PK would sensitize cancer cells upon treatment of chemotherapeutic drugs such as cisplatin. Together, our study described here not only validates DNA-PK as a useful molecular target for the treatment of drug-

resistant cancer cells, but also supports physiologic role for DNA-PK in IR or chemotherapy drug resistance of cancer cells.

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FIGURE LEGENDS

Figure 1. Panel A. A peptide co-therapy strategy for targeted inhibition of DNA-PK in cancer cell co-therapy. Treatment of cells with ionizing radiation (or chemotherapy drug) induces strand-break DNA damage. To repair DNA damage, DNA-PK heterotrimeric complex (Ku70, Ku80, and DNA-PKcs) needs to be assembled at the ends of DNA. Target peptide representing amino acids 720-732 of Ku80 not only interacts with DNA-PKcs, but may also be involved in Ku heterodimer assembly and DNA termini binding. As a result, cells treated with target peptide will exhibit poor or no DNA repair and become highly sensitive to ionizing radiation or chemotherapy drug. **Panel B.** Synthetic peptide used for co-therapy study. Sequences of synthetic peptide-based inhibitor and control peptides (single-letter amino acid code). The membrane-translocating hydrophobic signal sequence is indicated in *italic* letter and the nuclear localization sequence is shown in bold face. Twelve residue of peptide inhibitor region is indicated at the C-terminus of HNI-38.

Figure 2. Effect of the target peptide on interaction of Ku70/Ku80 with DNA-PK or with dsDNA. **Panel A.** Ku70/Ku80 complex is not in complex with DNA-PKcs. Chromatographic separation of DNA-PKcs from Ku70/Ku80 heterodimer. Partially purified DNA-PK fractions (dsDNA cellulose fraction; see Lees-Miller et al., 1990 for the details) were subjected to heparin-sepharose column chromatography and eluted with 100-500 mM NaCl gradient. Fractions were analyzed by 6% SDS-PAGE for DNA-PKcs and Ku80 followed by immunoblot using anti-DNA-PKcs and anti-Ku80 antibodies. **Panel B.** The target peptide (HNI-38) interferes with association of DNA-PKcs with dsDNA. Partially purified DNA-PK fraction (100 ng) was incubated with 0 nM (lane 2), 10 nM (lanes 3 & 6), 50 nM (lanes 4 & 7), and 100 nM (lanes 5 & 8) of either control peptide or target peptide prior to dsDNA cellulose pull-down assay. Lane 1 contained partially purified DNA-PK without dsDNA

pulldown assay. The protein-dsDNA cellulose complex was analyzed by the procedure described in Methods section. **Panel C.** Effect of HNI-38 on DNA binding activity of Ku70/Ku80 complex. Purified Ku70/Ku80 complex (100 ng) was incubated with 10 nM (lanes 4 & 7), 50 nM (lanes 3 & 6), and 100 nM (lanes 2 & 5) of either control peptide or target peptide prior to dsDNA cellulose pull-down assay. Following the dsDNA cellulose pull-down, Ku70 and Ku80 were analyzed by 10% SDS-PAGE and Western blot.

Figure 3. Effect of target peptide on DNA-PK kinase activity *in vitro*. Partially purified DNA-PK fraction was incubated with various concentrations of either control peptide or target peptide prior to the addition of substrate peptide and other components for DNA-PK kinase assay (see the Methods section for the details). DNA-PK activity was measured as the relative amounts of ^{32}P transferred to the substrate peptide.

Figure 4. Effect of target peptide on double-stranded DNA breaks (dsb) repair. Breast cancer cells (NCI) grown in ^{14}C -containing media were treated with ionizing radiation (40 Gy) in the presence of no peptide (lanes 1-6), 50 nM of control peptide (lanes 7-12), or 50 nM of target peptide (lanes 13-18). After harvesting the cells at various time points, intact chromosomes and double-stranded DNA breaks (DSBs) were separated by gel electrophoresis (**panel A**: fluorography) and were quantified by liquid scintillation counter (**panel B**).

Figure 5. Effect of control (HN-26) and target (HNI-38) peptides on the growth of breast cancer cells treated with ionizing radiation (**panel A**) or cisplatin (**panel B**). Values expressed are means (\pm S.E.) of the three replications (*, $p < 0.01$; ** $p < 0.01$). The clonogenic assay was used for the cells treated with ionizing radiation and the cell survival

assay (MTT) was employed for those treated with cisplatin (see the Methods section for the detailed procedure).

Figure 1A

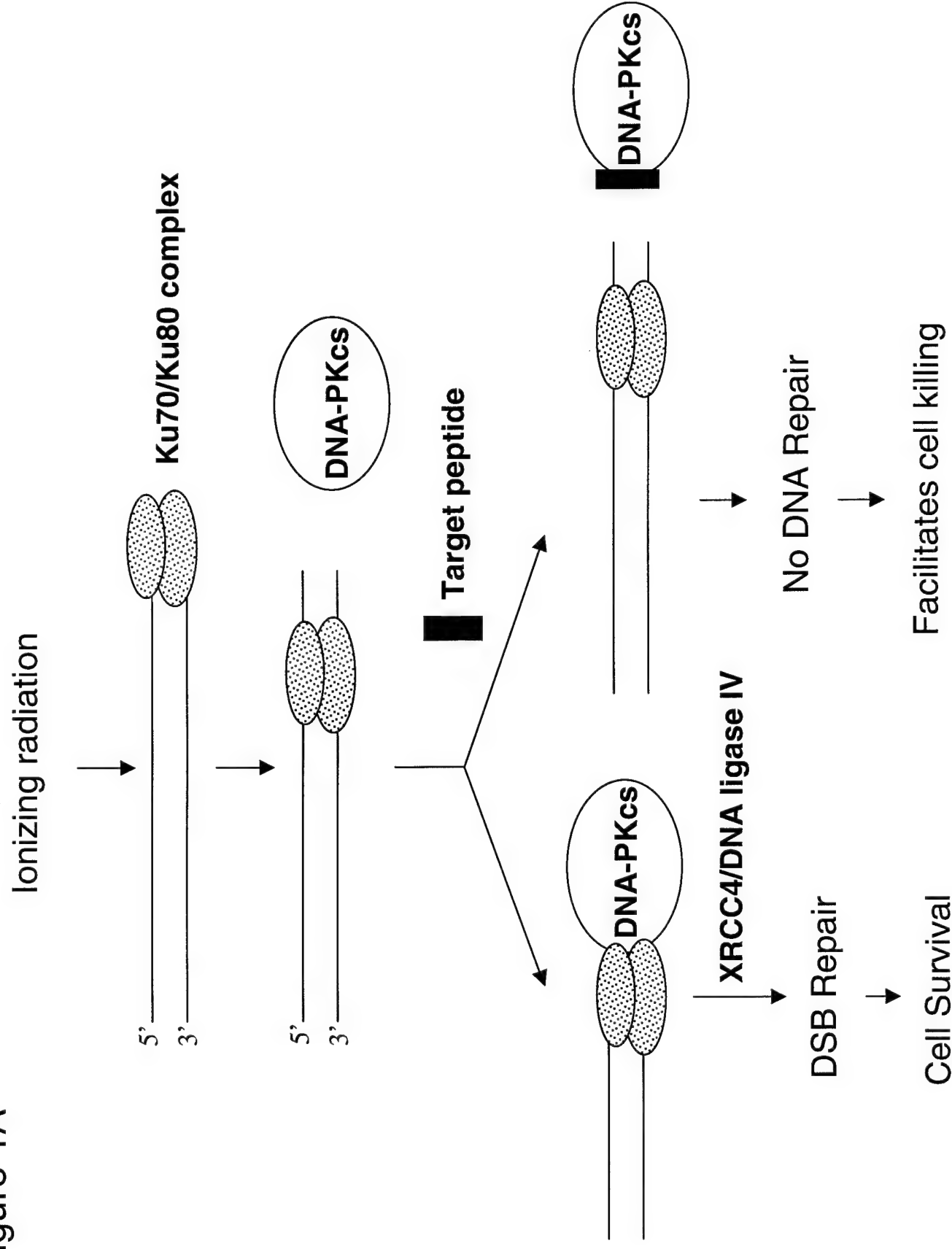


Figure 1B

HN-26

AAVALLPAVLLALLAPVQRKRQKLMY

HNI-38

AAVALLPAVLLALLAPVQRKRQKLMYEGGDVDDLLDMI

Figure 2A

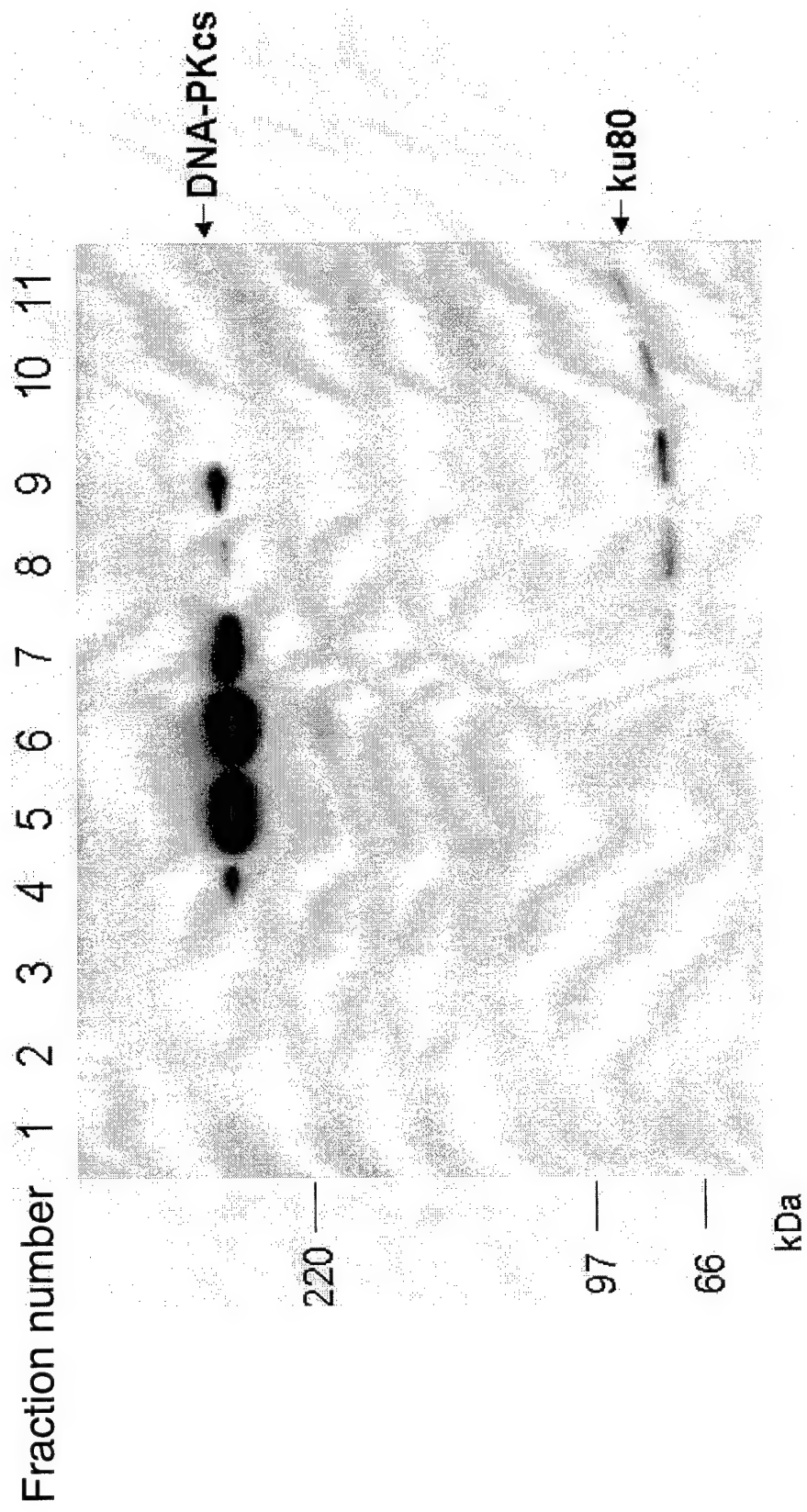


Figure 2B

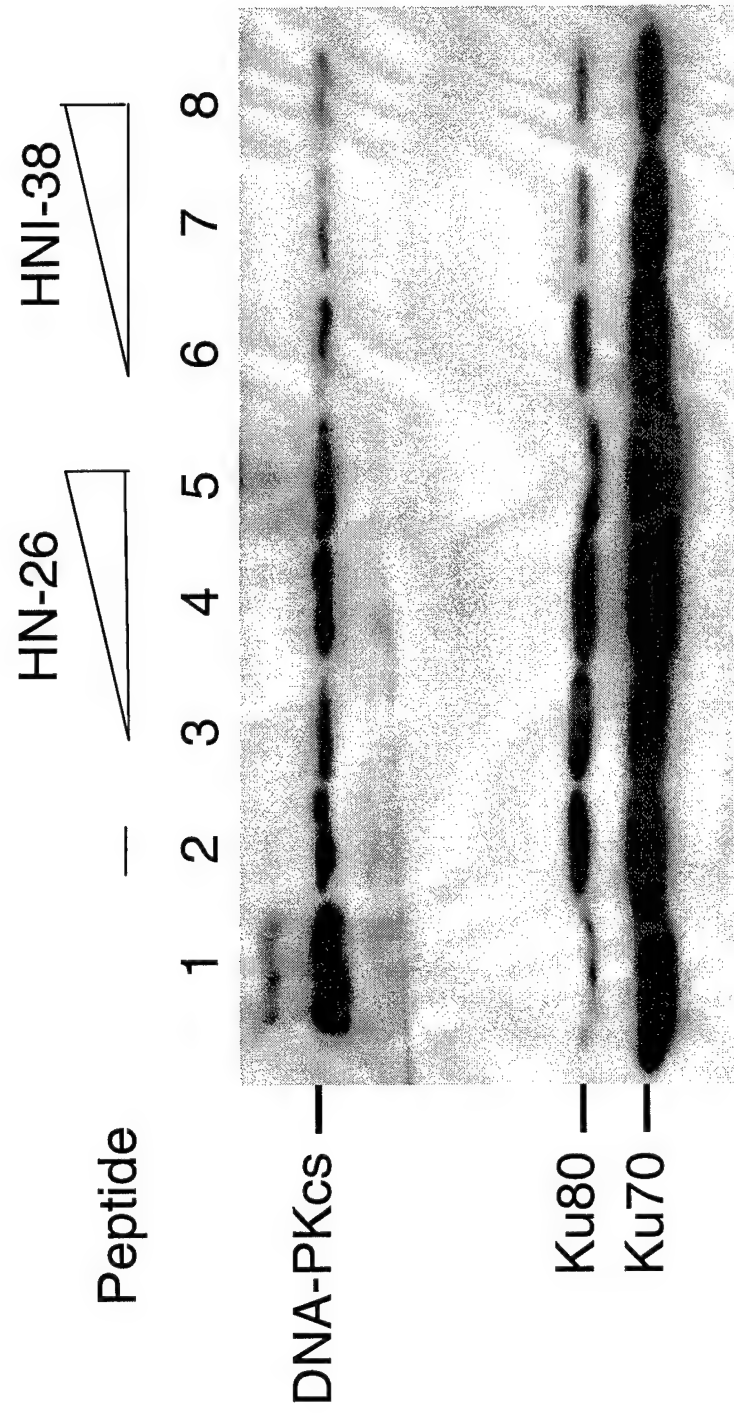


Figure 2C

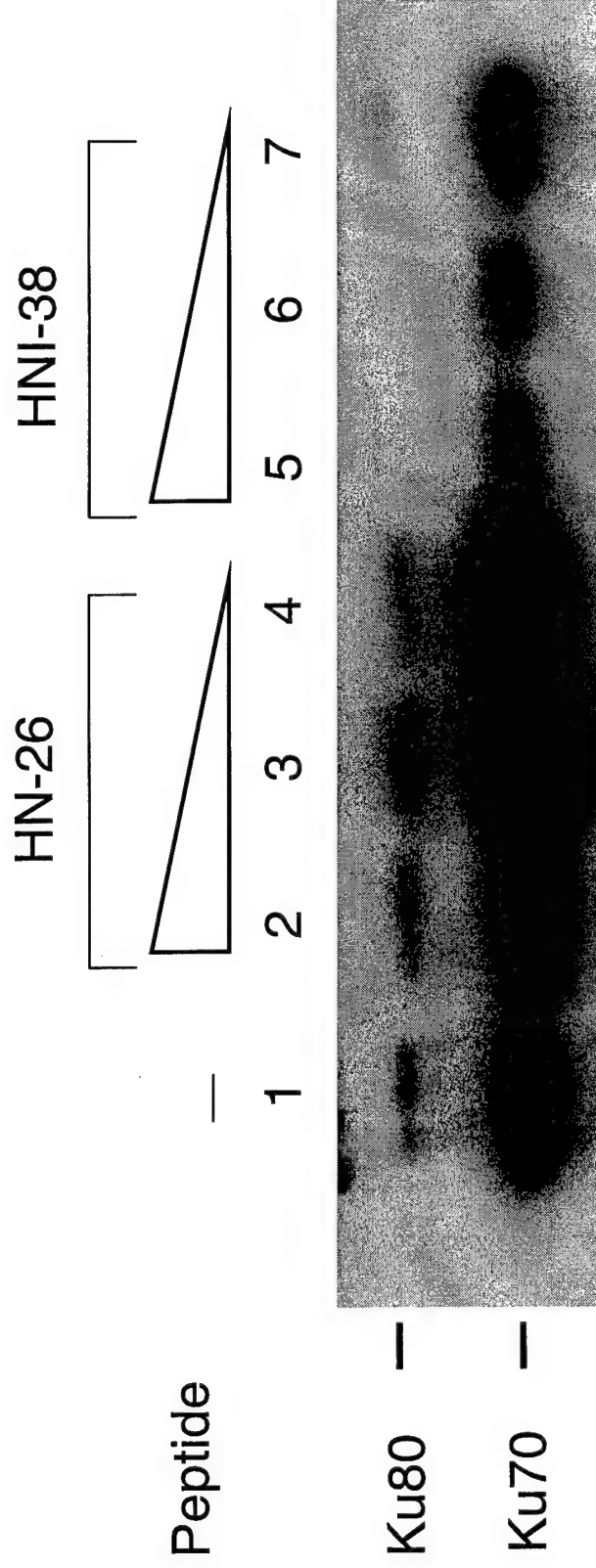


Figure 3

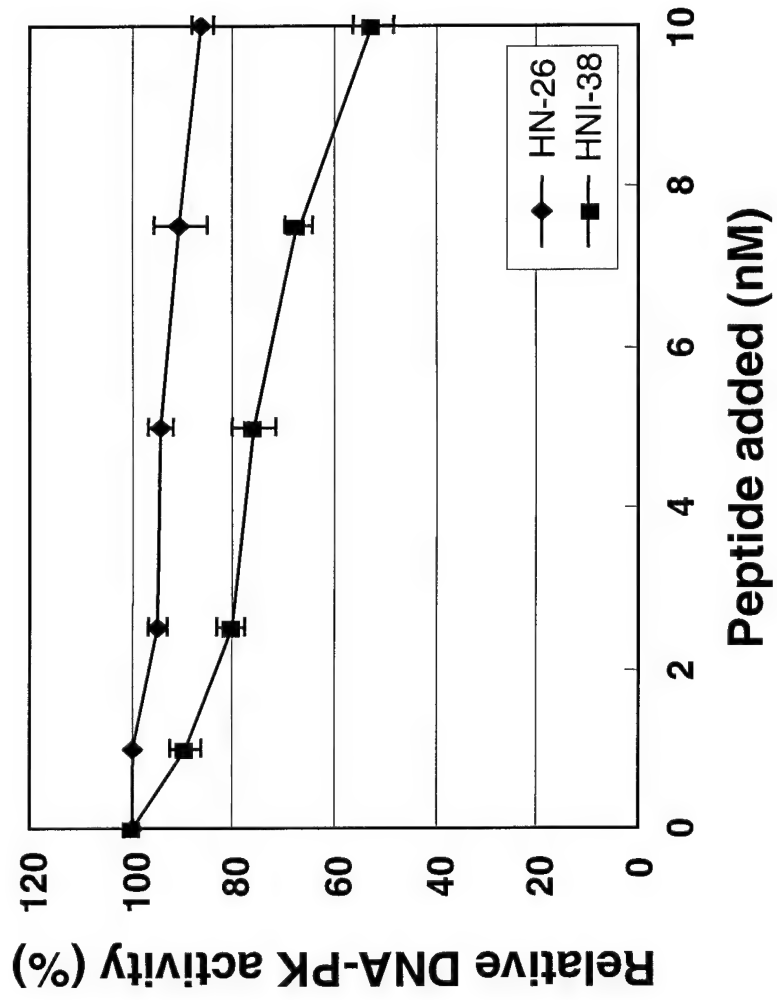


Figure 4A

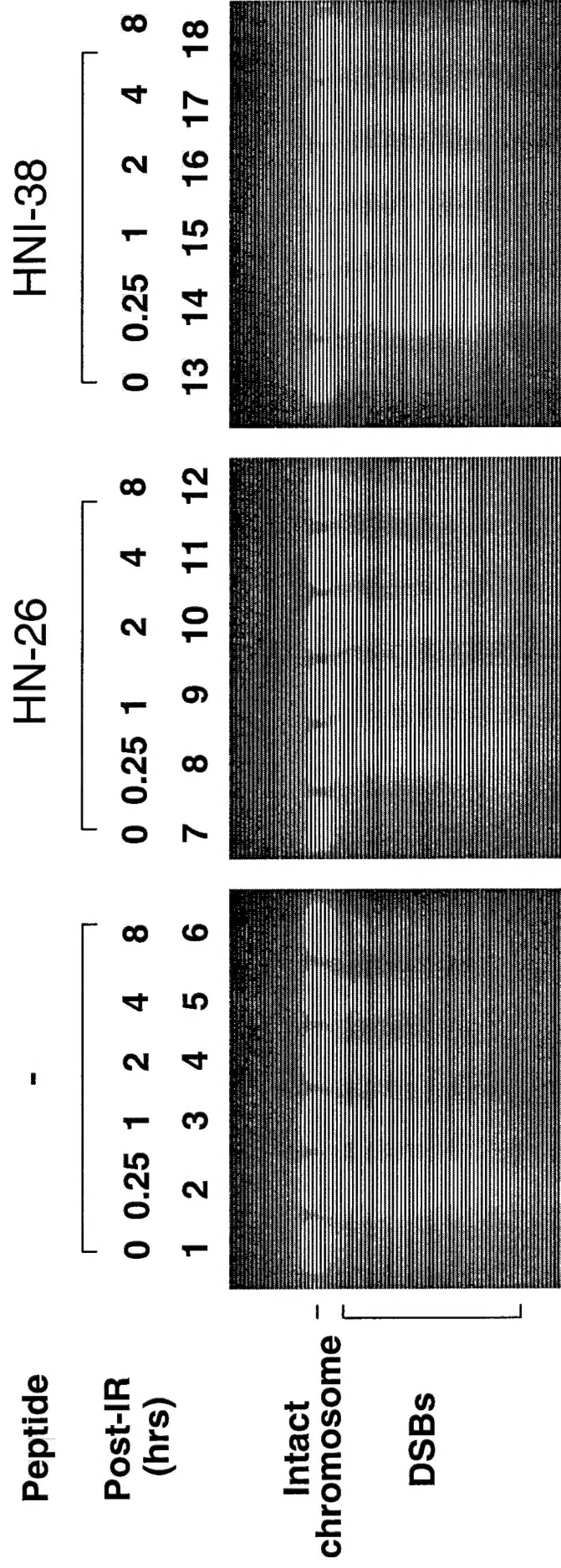


Figure 4B

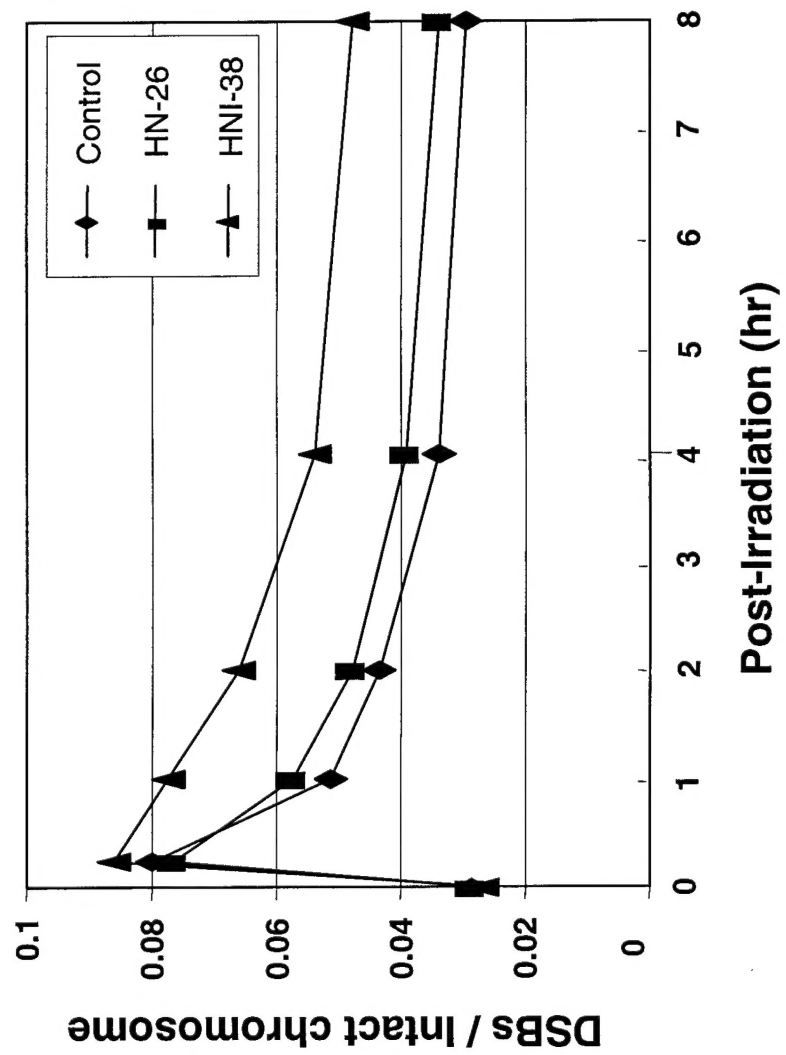


Figure 5A

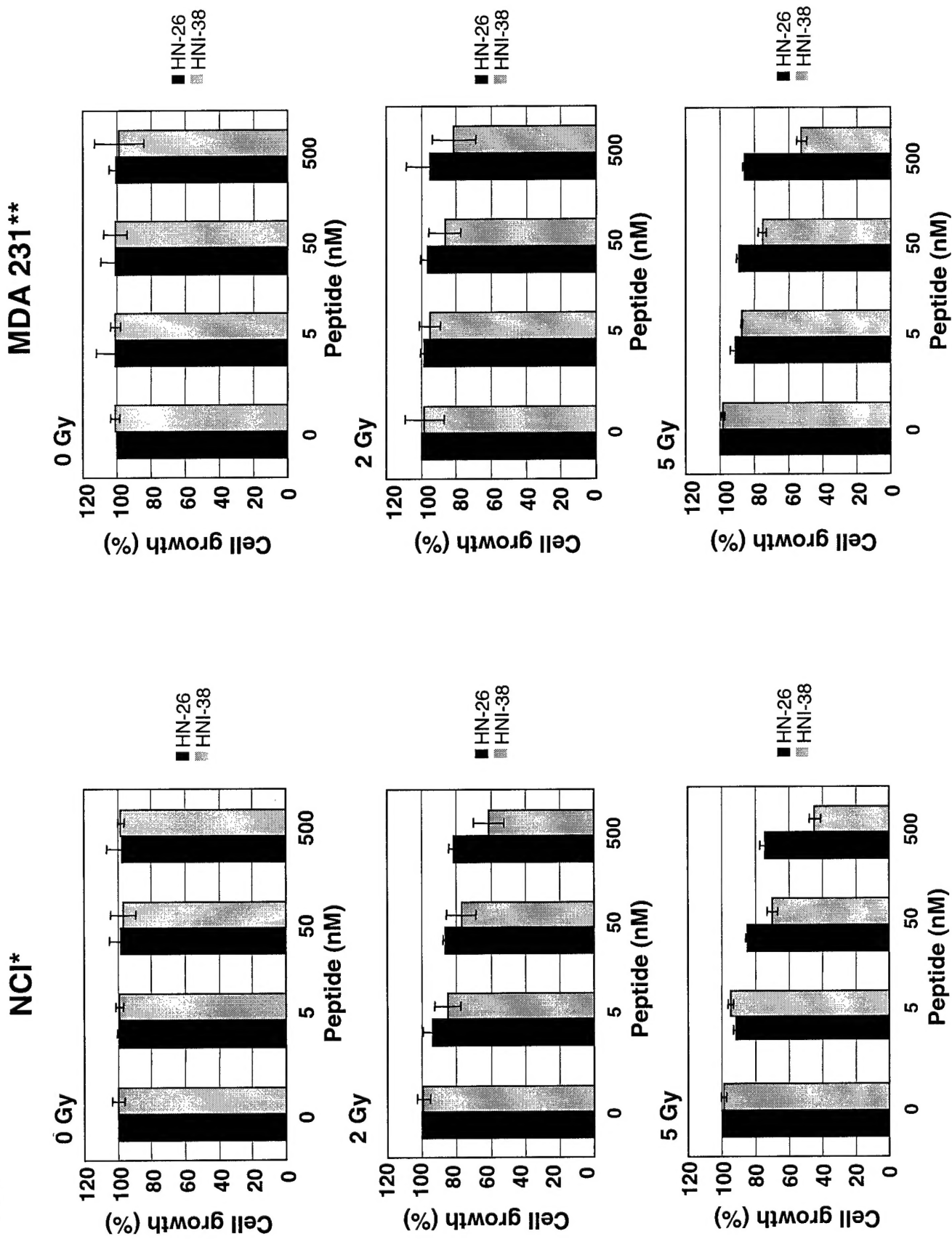


Figure 5B

